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Use of Alternative Primers for Gender Discrimination in Human Forensic Genotyping

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16. Abstract

An assay using the Federal Bureau of Investigation's human Combined DNA Identity System (CODIS) primers has been developed for polymerase chain reaction (PCR)-based human identity testing. Recent forensic literature has identified several human populations that carry a deletion mutation in the Y-chromosome copy of the amelogenin locus. This is the standard locus used for gender determination in CODIS. Additionally, the amelogenin male PCR products are very close in size requiring manual annotation of PCR electrophoresis results for this locus. This study was designed to test several gender-specific primers which are to loci outside the amelogenin region, have well-separated PCR products, and could serve as additions or replacements to amelogenin in our human identity testing assay.

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INTRODUCTION

Polymerase chain reaction-based human identity testing has been used successfully for genotyping of forensic samples. The Federal Bureau of Investigation developed a standardized set of loci and primers for use with PCR for genotyping. The Combined DNA Identity System (CODIS) contains a core set of STR markers for human identity testing[1] that are discriminatory over a wide range of ethnicities. An additional marker, amelogenin,[2] has been included in the CODIS series for gender discrimination.

The amelogenin primers are specific for a locus within the X-Y homologous region. Gender determination is based on the presence of an X copy and the presence or absence of a 6 base-pair shorter Y copy.[2] In 1998, Santos reported that two Sri Lankan individuals from 350 males from various ethnic groups carried a p-arm deletion of the Y chromosome which included the amelogenin region.[3] Thangaraj, in a 2002 study of 270 Indian males, found five carrying a similar deletion.[4] In a 2003 study of 338 individuals of Malay, Chinese, and Indian ethnicity Chang reported that four Indians and one Malay showed a deletion of the Y copy of the amelogenin locus.[5] There are additional reports of amelogenin Y nulls in an Australian Caucasian,[6] a Moroccan father-son pair,[7] and six Austrians.[8] Chang concludes that there appears to be an amelogenin failure as high as 3.6% in the Malaysian Indian population and a lower but noticeable deficit of 0.02% in the Caucasian population.[5]

Recently, we reported the development of a genotyping protocol based on the use of primers from the CODIS set, including the gender determining marker, amelogenin,[9] using microfluidics rather than capillary electrophoresis for amplicon detection. Based on the low but significant probability of misidentifying samples, we considered it advantageous to our genotyping protocol to develop an additional gender determination assay for use in suspected Y nulls. Additionally, we were interested in developing an assay that could potentially replace or be an addendum to the amelogenin assay since the very close size difference between the X and Y allele products was sometimes difficult for microfluidics instruments to automatically detect, thus requiring manual annotation.

Additional genotyping assays have been developed for a series of Y-specific markers, which include a series of short tandem repeat regions in the DYS locus found on the q-arm of the Y chromosome (see, for example, studies done by Butler[10] and Ruitberg[11]). Gilson reported the use of a mixture of primers for two loci that can be used in a wide variety of mammals including humans for sex determination.[12] One primer pair is designed to a region of the Y-specific gene SRY,[12,13] and the second is to the homologous zinc finger protein genes of the X and Y chromosomes, ZFX/ZFY.[14] The successful use of these primers in humans has been reported.[15]

The current study took advantage of the availability of human forensic samples to test alternative gender determining primers. We tested Y-specific short tandem repeat primer sets for loci DYS390,[10] DYS438, and DYS439,[16] all q-arm loci, and the primers for p-arm loci ZFX/ZFY and SRY. The results were compared to amelogenin for confirmation of gender.

MATERIALS AND METHODS

Sample sets

Four samples were used for characterization studies. Two male samples, 04010722-1 from liver, and 03010577-11 from blood, were forensic in origin. Samples 23A and 23B were blood from female volunteers, collected under a local Internal Review Board protocol for developmental work. After initial characterization of the primers, 12 additional forensic samples were used to validate the selected conditions across a variety of tissue types and DNA quality (Table 1).

Genomic DNA extraction

We stored and processed samples as described in a previous report.[9] Briefly, tissue and blood were stored at -20° C. Approximately 25 mg of tissue was minced and processed with the ChargeSwitch gDNA Mini Tissue kit, #CS11204, using the manufacturer's directions (Invitrogen; Carlsbad, CA). DNA from whole blood was extracted from 200ul whole blood using the QIAamp DNA Mini Kit #51304 (Qiagen, Valencia, CA).

PCR primers and amplification

The sequences for and expected product size of the six primer pairs used in the study are shown in Table 2. All primers were synthesized commercially (Integrated DNA Technologies; Coralville, IA). A mix of ZFX/ZFY and SRY primers for initial testing was provided by Brandt Cassidy, DNA Solutions; Oklahoma City, OK.

Reaction setup was for 25ul final volume. Each reaction contained 5ng template, 400-800nM primers, 2.5mM dNTPs, 0.1% TritonX-100, 1x Amplitaq Gold buffer, and 2.25units Amplitaq gold. Amplification was as previously reported.[9] Thermal cycling was performed on a GeneAmp 9600 thermal cycler (PerkinElmer; Wellesley, MA) using the following cycling profile for 40 cycles:

An initial 95° C 11 min, 96° C 1 min; then 94° C 30sec, ramp 68 sec to 60° C, hold for 30 sec, ramp 50 sec to 70° C, hold for 45 sec, for 10 cycles. Then 90° C 30 sec, ramp 50 sec to 70° C, hold for 45 sec, for 30 cycles. Then 60° C for 30 min, 4° soak.

Electrophoresis

Detection of a 1ul aliquot from a 1:3 dilution in distilled water of each amplification product was performed on a 2100 Bioanalyzer using Agilent Expert software version B.02.03.SI307 and DNA 1000 Series II Labchip kits #5067-1504 (Agilent Technologies; Palo Alto, CA).

Table 1. Additional forensic samples used in the study.

Sample ID	Source
03010577-1	blood
03010577-7	liver
03010577-11	blood
08010522-1	blood
08010522-3	heart
08010522-5	brain
01010622-1	liver
01010622-3	kidney
04010622-4	kidney
08010622-1	blood
04010722-1	kidney
04010722-2	skin

RESULTS AND DISCUSSION

PCR was performed with the four characterization samples using DYS primers at 800nM and mixture primers at 400nM under standard PCR conditions (see Materials and Methods section). DYS390 and DYS439 gave the expected product for the two males and were negative for female samples. DYS438 showed the expected single peak with the male samples but showed three products with the female. Due to the presence of these spurious products, DYS438 was eliminated from further testing. A mixture of ZFX/ZFY and SRY primers received from Brandt Cassidy was tested at 400nM with the four samples and gave the expected product results, a single peak for females and two peaks for males (see Table 2 for expected products and sizes).

The criteria for a reliable gender determination test were the ability to detect both males and females with a differential signal for each, as seen with amelogenin.

Table 2. Primers used in study.

Locus	Primer Sequence	Product Size or Range (bp)	Gender	Reference
Amelogenin	ACC TCA TCC TGG GCA CCC TGG TT AGG CTT GAG GCC AAC CAT CAG	212(Y) or 218(X)	X and Y	Mannucci (2)
DYS390	TAT ATT TTA CAC ATT TTT GGG CC GTG ACA GTA AAA TGA AAA CAT TGC	189-233	Y-specific	Butler (10)
DYS438	TGG GGA ATA GTT GAA CGG TAA GTG GCA GAC GCC TAT AAT CC	202-242	Y-specific	Ayub (16)
DYS439	TCC TGA ATG GTA CTT CCT AGG TTT GCC TGG CTT GGA ATT CTT TT	236-256	Y-specific	Ayub (16)
SRY	CCC ATG AAC GCA TTC ATT GTG TGG ATT TTA GCC TTC CGA CGA GGT CGA TA	224	Y-specific	Gilson (12)
ZFX/ZFY	GCA CTT CTT TGG TAT CTG AGA AAG T ATA ATC ACA TGG AGA GCC ACA AGC T	445	X and Y	Aasen (14)

This ruled out the use of the DYS or SRY primers, alone, since they are Y-specific. Therefore, three primer mixes, each containing the ZFX/ZFY primer pair, which gives a characteristic 445 bp product with a female sample, and one male-specific primer pair were tested. The results were compared to amelogenin, which gives a single peak for a female template, a doublet peak with a male. The mixes, all at 800nM for each primer, were DYS390 + ZFX/ZFY, DYS439 + ZFX/ZFY, and SRY + ZFX/ZFY. The last mix, SRY+ZFX/ZFY, is similar to the one received from Brandt Cassidy but at 800nM for each primer, rather than 400nM (Figure 1).

From Figure 1A, it is clear that the small difference in size of the X versus Y chromosome amelogenin product results in a doublet rather than the well-separated peaks as seen in Figure 1B-D for male samples with the three primer mixes. Note that the ZFX/ZFY products seen for the female sample have a higher signal than the male since there are two copies of the X chromosome present. Also note that secondary products are present for the male samples for all Y-specific primers tested, including amelogenin. All three mixes gave distinct peaks for male samples. There was, however, a differential level of signal between the three Y-specific primer products and ZFX/ZFY. ZFX/ZFY showed a fluorescent unit (FU) measure on the Bioanalyzer of roughly 100 FU at 800nM with the male samples. But the Y-specific primers ranged from a high of 100 FU with SRY to 40 FU with DYS390 to a low of less than 40 FU with DYS 439 (Figure 1B-D). The amplitude of secondary products with DYS and SRY primers were all in the range of 5-15 FU. The SRY + ZFX/ZFY mix was determined to have optimal performance of the three primer mixes showing the highest overall signal intensities of products, the most closely equivalent product signal intensities, and lowest secondary product signal strengths.

We attempted to reduce the secondary products presence by reducing the primer concentration in the SRY + ZFX/ZFY mix. A range of 400, 600, and 800nM was examined for product and secondary product peak strength with the same four samples. No secondary products were detected at 400nM, but signal intensity was reduced to 15 FU. Increasing SRY concentration to 600 and 800nM, respectively, showed the presence of the secondary product doublet at less than 5 FU for 600nM and 5-8 FU 800nM. The SRY amplicon peak height at 600nM averaged 50 FU and 70 FU at 800nM (Figure 2). The relatively low level of secondary product and highest product FU at 800nM suggested that this was the optimal concentration. The difference in

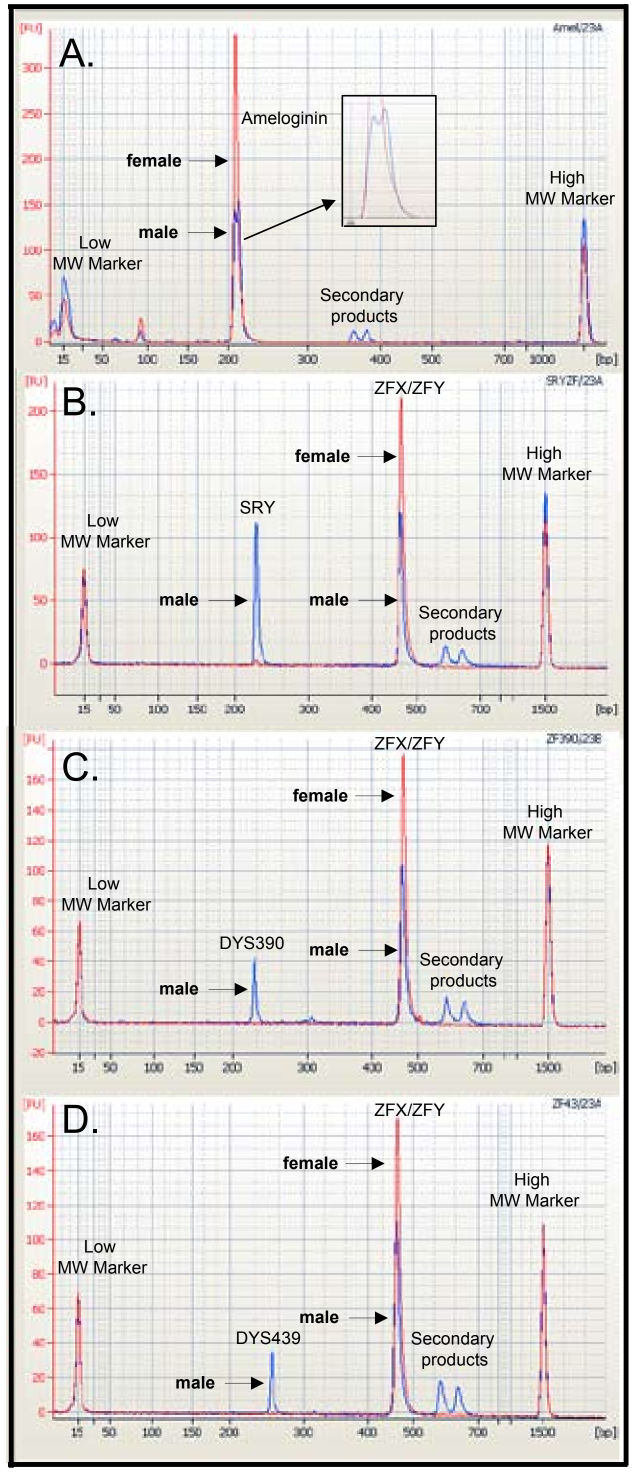

Figure 1. Gender primer mix PCR products. Shown are overlays of bioanalyzer results of the PCR products from the three mixes and amelogenin (see Results and Discussion section). Male sample 04010722-1 and female sample 23A (taller peaks in Amelogenin and ZFX/ZFY overlays) are compared. A. Amelogenin; insert is a close-up of the characteristic male doublet. B. SRY+ZFX/SFY C. DYS390+ ZFX/SFY D. DYS439+ ZFX/SFY.

signal intensity seen in the two SRY PCR series using 800nM primers with the same templates suggests that the variations between experiments may result in signals ranging from 70-100 FU for the SRY products and 5-15 for the secondary product doublet. These levels are acceptable for detection on the Bioanalyzer in the case of the product and remain below the default cutoff for the secondary products.

Twelve forensic samples were tested with the mix at 800nM to assure consistent results across a variety of samples. These samples had been previously tested by PCR with amelogenin. All the samples appeared male, based on amelogenin results, except 04010722-2, which gave a negative amelogenin result (data not shown). Four samples were from blood, and eight were from a variety of tissues (Table 1). Eleven of the samples showed the expected peaks for SRY and ZFX/ZFY at signal strengths ranging from 50-100 FU. Sample 04010722-2 showed a very weak signal, a single peak for SRY with 10 FU but no ZFX/ZFY peak. This was not surprising since this sample had been negative with amelogenin. The absence of the ZFX/ZFY peak, which represents an amplicon of 445bp compared to the weak 245bp SRY amplicon, suggests sample degradation. Furthermore, the presence of a peak from the SRY locus unambiguously determines that the sample is from a male, regardless of the presence or absence of a signal from the X-determinant locus. Overall, the gender mix performs satisfactorily with forensic samples, giving expected results with DNA isolated from both tissue and blood.

In summary, the availability of additional gender determination loci will expand our repertoire of robust primers for use in forensic sample identification. This is important because of the null-Y mutation that results in a deletion of the amelogenin gene located at 6.79Mbp on the Y chromosome.[3 4 5] The mutation occurs especially within certain Indian populations but has also been found in Caucasian populations.[6 7 8] The result of this deletion is the incorrect identification of the individual as female when using amelogenin as the gender-determining locus. The extent of the mutation has been estimated. The MSY1 locus, a Y-specific mini-satellite marker on the same p-arm as amelogenin, but less than a Mbp away, was negative with an amelogenin deletion group.[5] Subjects carrying the null-Y marker had a Y-chromosome deletion of about 1Mbp.[4] Finally, the TSPYP1 locus, located at 6.19Mbp, was found to be a proximal endpoint of the deletion.[3] The STY and ZFY genes are on the p-arm of the Y chromosome at 2.56Mbp and 2.86 Mbp respectively. Both are significant distances from the amelogenin gene deletion region and therefore are good candidates for alternative gender determination loci.

Certainly, in human forensic cases where there is a possibility of an amelogenin deletion, it would be necessary to use an alternative locus. However, it also will be very helpful to use the ZFX/ZFY and SRY primer mix to facilitate automatic annotation of the Bioanalyzer results because of the excellent separation and signal strength of the products.

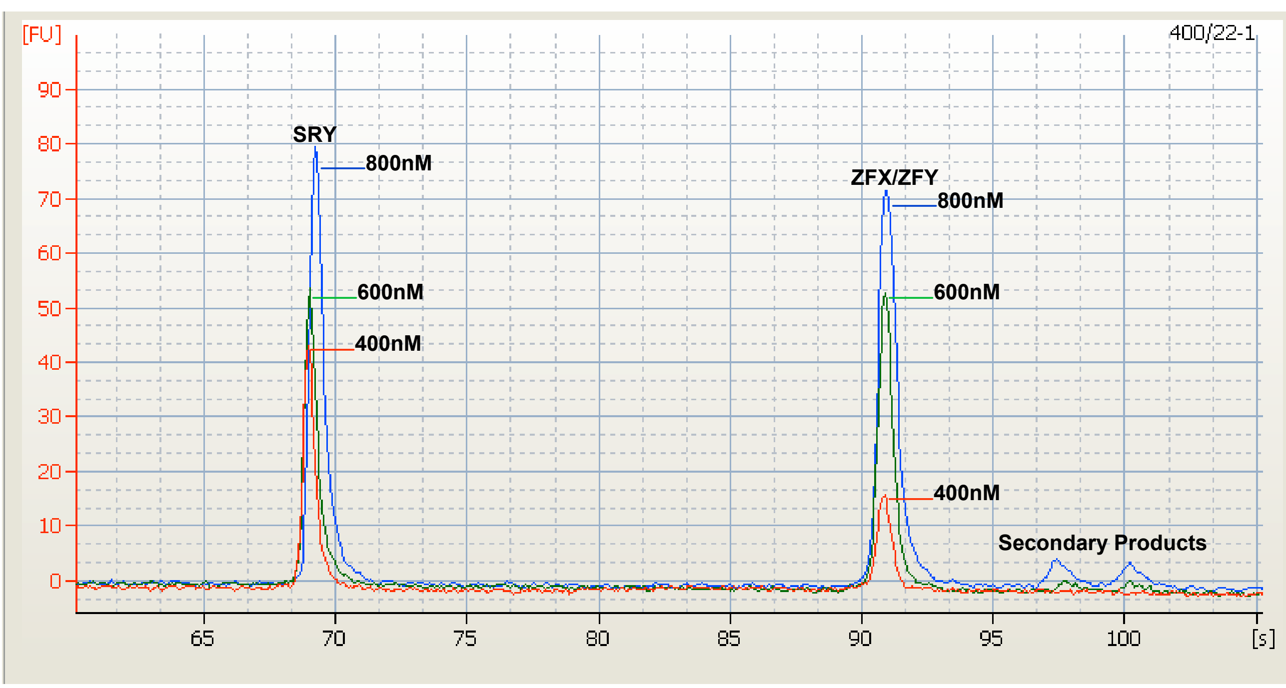

Figure 2. Comparison of PCR product signal strengths with SRY +ZFX/ZFY primer mixes. The product peaks are from male sample 04010722-1. The concentration of primers in the mix is shown.

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